Critical developmental period for hyperoxia-induced blunting of hypoxic phrenic responses in rats

R. W. BAVIS,1 E. B. OLSON, JR.,2 AND G. S. MITCHELL1
Departments of 1Comparative Biosciences and 2Preventive Medicine, University of Wisconsin, Madison, Wisconsin 53706

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Bavis, R. W., E. B. Olson, Jr., and G. S. Mitchell. Critical developmental period for hyperoxia-induced blunting of hypoxic phrenic responses in rats. J Appl Physiol 92: 1013–1018, 2002. First published November 2, 2001; 10.1152/japplphysiol.00859.2001.—Hypoxic ventilatory and phrenic responses are reduced in adult rats reared in hypoxia (60% O2) for the first month of life but not after hyperoxia as adults. In this study, we identified the developmental window for susceptibility to hyperoxia. Phrenic nerve responses to hypoxia were recorded in anesthetized, vagotomized, paralyzed, and ventilated Sprague-Dawley rats (aged 3–4 mo) exposed to 60% O2 for the first, second, third, or fourth postnatal week. Responses were compared with control rats and with rats exposed to 60% O2 for the first month of life. Phrenic minute activity (burst amplitude × frequency) increased less during isocapnic hypoxia (arterial P O2 = 60, 50, and 40 Torr) in rats exposed to hypoxia for the first or second week, or the first month, of life (P < 0.01 vs. control). Functional impairment caused by 1 wk of hyperoxia diminished with increasing age of exposure (P = 0.005). Adult hypoxic phrenic responses are impaired by 1 wk of hyperoxia during the first and second postnatal weeks in rats, indicating a developmental window coincident with carotid chemoreceptor maturation.

respiratory control; control of breathing; developmental window; carotid body

ENVIRONMENTAL CONDITIONS DURING early life profoundly influence the development of vertebrate neural systems (19), including neural pathways associated with the control of breathing. Similar conditions often have no effect, or only transient effects, in adult animals, suggesting that such plasticity results from the environment experienced during critical periods of development. For example, mature humans and other mammals sojourning at high altitude gradually increase their resting ventilation and hypoxic sensitivity, and these changes reverse on return to sea level (5, 35). However, some high-altitude residents acquire blunted hypoxic ventilatory responses during development that may remain attenuated at sea level (30, 40). Recent studies confirm that the respiratory environment during development can have long-lasting effects on vertebrate respiratory control (2, 21, 24, 31, 33, 34, 37, 38, 42, 44). Indeed, perinatal hypoxia in rats and sheep may diminish hypoxic ventilatory responsiveness for periods lasting weeks to months (33, 38).

Chronic hyperoxia during development also impairs subsequent hypoxic ventilatory responses (13, 22). Rats exposed to 60% O2 for the first month of life exhibit dramatic reductions in their adult hypoxic ventilatory response, whereas similar hypoxic exposures in adults do not cause functional deficits (21). Thus these effects of hyperoxia are limited to development. Subsequent studies demonstrated significant reductions in phrenic nerve responses to hypoxia in anesthetized, paralyzed, vagotomized, and artificially ventilated rats, ruling out changes in respiratory mechanics or gas exchange as causes of this functional impairment (24). Rather, developmental hyperoxia disrupts normal carotid body function (7, 9, 13, 22) without appreciably altering the central integration of inputs from carotid chemosensitive neurons (23).

One week of 60% O2 during early postnatal development is sufficient to produce morphological changes in the carotid body similar to those observed after 1 mo of hypoxia (9). Thus shorter exposures to hyperoxia during development may cause functional impairment of peripheral chemoreceptors. In the present study, we defined (with 1-wk resolution) the critical developmental period for hyperoxia-induced blunting of hypoxic phrenic responses. Separate groups of rat pups were exposed to 60% O2 for 1-wk periods during the first through fourth weeks of life, and their phrenic responses to isocapnic hypoxia were assessed as young adults.

METHODS

All experimental procedures were approved by the Animal Care and Use Committee of the School of Veterinary Medicine at the University of Wisconsin-Madison.

Experimental groups. Experiments were conducted on six groups of adult (3–4 mo old), male Sprague-Dawley rats (colony 236b, Harlan Sprague Dawley, Madison, WI). “Hyperoxia-treated” rats were exposed to 60% O2 (cf. Ref. 21) from 2–5 days before birth through their first 30 postnatal days (1 mo; n = 8 rats/3 litters; 368 ± 7 g), from 2–3 days before birth through their first 7 postnatal days (week 1; n =...
8/3 litters; 365 ± 10 g), during postnatal days 8–14 (week 2; \( n = 8/3 \) litters; 402 ± 8 g), during postnatal days 15–21 (week 3; \( n = 8/2 \) litters; 388 ± 11 g), or during postnatal days 22–28 (week 4; \( n = 8/2 \) litters; 392 ± 12 g). Hyperoxia-treated rats were housed in normoxic conditions at all other times. Age-matched control rats (\( n = 8/3 \) litters; 371 ± 7 g) were born and raised in the same room as hyperoxia-treated rats but were exposed to normoxic conditions throughout development. Because only one environmental chamber was available for this study, we were unable to expose control rats to chamber conditions while maintaining a randomized, blinded experimental design. Nevertheless, during hyperoxic exposures, chamber temperature, humidity, and CO2 were similar to those outside the chamber.

**Experimental preparation.** Anesthesia was induced with isoflurane and maintained [2.5% isoflurane; inspired O2 fraction (\( \mathrm{FiO}_2 \)] = 0.5-balance \( \mathrm{N}_2 \] first through a nose cone and then via a tracheal cannula placed to allow pump ventilation (model 683 rodent respirator, Harvard Apparatus, Holliston, MA). A catheter was placed in the femoral vein, and the rats were then hyperoxygenated to urethane anesthesia (1.6 g/kg iv). Adequacy of anesthesia was monitored throughout the experiment by testing blood pressure responses to toe pinch; supplemental urethane was given if a response was observed. A catheter was placed in the femoral artery to monitor blood pressure and to withdraw 0.2-ml blood samples to determine blood gases and pH with a blood analysis system (model ABL-500, Radiometer, Copenhagen, Denmark). Arterial blood values were corrected to rectal temperature, which was maintained at 37–38°C with a heated table. Animals received a continuous intravenous infusion (2.5 ml/h) of a 5% sodium bicarbonate-lactated Ringer solution (1:11 vol/vol) to maintain fluid and acid-base balance, beginning shortly after urethane administration. Animals were bilaterally vagotomized in the midcervical region and paralysed with pancuronium bromide (3.25–3.5 mg/kg) to prevent spontaneous breathing movements and entrapment of neurograms with the ventilator. End-tidal CO2 partial pressures (\( \mathrm{PETCO}_2 \)) were measured with a rapidly responding flow-through \( \mathrm{CO}_2 \) analyzer (Capnogard, Novametrix, Wallingford, CT) placed in the expirited line of the ventilator circuit.

The left phrenic nerve was isolated by using a dorsal approach, cut distally, desheathed, submerged in mineral oil, and placed on a bipolar silver wire electrode. Nerve activity was amplified (×10,000), band-pass filtered (100 Hz to 10 kHz; model 1800, A-M Systems, Carlsborg, WA), and integrated (time constant = 50 ms; model MA-821RSP moving averager, CWE, Ardmore, PA). The integrated signal was digitized and processed with commercially available computer software (WINDAQ, Dataq Instruments, Akron, OH).

**Experimental protocol.** Preparations were allowed to stabilize for ~60 min after surgery (\( \mathrm{FiO}_2 = 0.5, \mathrm{PETCO}_2 \) = 40 Torr). The \( \mathrm{CO}_2 \) apneic threshold was then determined by hyperventilating the rat until phrenic nerve activity ceased and then slowly raising \( \mathrm{PETCO}_2 \) by decreasing ventilator rate or increasing inspired \( \mathrm{CO}_2 \), until rhythmic activity reappeared. Baseline neural activity was standardized among preparations by maintaining \( \mathrm{PETCO}_2 \) 3 Torr above the \( \mathrm{PETCO}_2 \) at which activity resumed.

After establishment of baseline phrenic nerve activity (20–30 min), an arterial blood sample was drawn; subsequent blood samples were compared with this baseline value. Isocapnic hypoxic responses were assessed at three target arterial \( \mathrm{PO}_2 \) (\( \mathrm{PaO}_2 \)) levels (60, 50, and 40 Torr). Each hypoxic episode lasted 5 min, and arterial blood samples were collected during the final 30 s of the exposure. Trials were accepted if \( \mathrm{PaO}_2 \) was ± 2 Torr of the target \( \mathrm{PaO}_2 \) and arterial \( \mathrm{PCO}_2 \) (\( \mathrm{PaCO}_2 \)) was ± 2 Torr of baseline; experiments usually required three to five hypoxic trials to obtain data that fit these criteria at all three \( \mathrm{PaO}_2 \) levels. Between trials, rats were returned to \( \mathrm{FiO}_2 = 0.5 \) for 5–10 min. After the final hypoxic challenge, the "maximal" phrenic response to hypercapnia was measured by increasing \( \mathrm{PETCO}_2 \), 85–90 Torr. At the conclusion of experiments, rats were killed via urethane overdose.

**Data analysis.** A blinded design was used to conceal the identity of rats from the investigator during data collection and preliminary analysis. Phrenic activity was averaged in 30-s bins (immediately preceding blood sampling) under baseline conditions, during the fifth minute of hypoxia, and at the end of the hypercapnic challenge. Variables measured included peak amplitude of integrated phrenic activity, phrenic burst frequency (in bursts/min), and their product, minute phrenic activity. Changes from baseline in burst amplitude and minute activity were normalized as a percentage of baseline phrenic activity (% baseline) and as a percentage of phrenic activity during hypercapnia (% maximum). This dual-normalization procedure minimizes concerns about potential normalization artifacts that can arise when neurograms are compared within and among experiments (10). Because overall conclusions were unaffected by the normalization method, only data expressed as a percentage of baseline are reported. We were unable to detect litter effects for hypoxic phrenic responses in this study, or in previous studies using similar experimental methods and design (e.g., Ref. 12), so data were pooled across litters within each treatment group. Hypoxic responses and blood gases were compared among treatment groups by using a two-way repeated-measures ANOVA followed by Student-Newman-Keuls post hoc tests or by regression (SigmaStat 2.03, SPSS, Chicago, IL). Differences were considered significant at \( P < 0.05 \). All data are presented as means ± SE.

**RESULTS**

**Blood gases.** Mean \( \mathrm{PaO}_2 \) did not differ among treatment groups (\( P > 0.05 \)) and was within 1 Torr of the target \( \mathrm{PaO}_2 \) at each level of hypoxia (Table 1). \( \mathrm{PaCO}_2 \) was 2–3 Torr lower in week 1 and 1-mo hyperoxia-treated rats relative to untreated control rats (\( P = \)

| Table 1. Blood-gas values during baseline conditions and during isocapnic hypoxia in control rats and rats exposed to hypoxia during development |
|---|---|---|---|
| **Group** | **Level of Hypoxia** | **Baseline** | **60 Torr** | **50 Torr** | **40 Torr** |
| **PaO2, Torr** | | | | | |
| Control | 233 ± 8 | 60 ± 1 | 49 ± 1 | 40 ± 1 |
| 1 Mo | 250 ± 4 | 61 ± 1 | 49 ± 1 | 40 ± 1 |
| Week 1 | 232 ± 5 | 60 ± 1 | 49 ± 1 | 40 ± 1 |
| Week 2 | 230 ± 5 | 60 ± 1 | 49 ± 1 | 40 ± 1 |
| Week 3 | 226 ± 10 | 60 ± 1 | 51 ± 1 | 40 ± 1 |
| Week 4 | 229 ± 6 | 60 ± 1 | 49 ± 1 | 40 ± 1 |
| **PaCO2, Torr** | | | | | |
| Control | 48 ± 1 | 47 ± 1 | 48 ± 1 | 49 ± 1 |
| 1 Mo | 45 ± 1* | 45 ± 1* | 46 ± 1* | 46 ± 1* |
| Week 1 | 45 ± 1* | 44 ± 1* | 45 ± 1* | 46 ± 1* |
| Week 2 | 46 ± 1 | 45 ± 1 | 47 ± 1 | 47 ± 1 |
| Week 3 | 46 ± 1 | 46 ± 1 | 46 ± 1 | 47 ± 1 |
| Week 4 | 47 ± 1 | 46 ± 1 | 47 ± 1 | 47 ± 1 |

Values are means ± SE for 8 rats per group. \( \mathrm{PaO}_2 \), arterial \( \mathrm{PaO}_2 \); \( \mathrm{PaCO}_2 \), arterial \( \mathrm{PaCO}_2 \). *\( P < 0.05 \) compared with control rats.
Phrenic response to hypoxia. Changes in phrenic amplitude during hypoxia differed significantly among treatment groups (P < 0.001), with phrenic amplitude increasing one-third to one-half as much in week 1, week 2, and 1-mo hyperoxia-treated rats vs. untreated control rats (all P < 0.02; Fig. 1). Phrenic amplitude hypoxic responses were also reduced in week 1 hyperoxia-treated rats compared with week 4 rats (P = 0.007), whereas 1-mo hyperoxia-treated rats had only marginally reduced responses vs. week 4 rats (P = 0.075). There were no other statistically significant differences among groups.

Increases in phrenic burst frequency during hypoxia also differed among treatment groups (P = 0.004), primarily due to smaller increases in burst frequency in week 1 and 1-mo hyperoxia-treated rats (both P = 0.006) vs. week 3 hyperoxia-treated rats (Fig. 1). Increases in burst frequency in week 1 and 1-mo hyperoxia-treated rats tended to be smaller than in other treatment groups as well, but these differences did not reach statistical significance (all P > 0.05).

As a result of smaller increases in phrenic amplitude, and somewhat smaller increases in burst frequency, hypoxia-induced increases in minute phrenic activity were also considerably reduced in week 1, week 2, and 1-mo hyperoxia-treated rats vs. untreated control rats (all P < 0.01; Fig. 1). Similarly, increases in minute activity during hypoxia for week 1 and 1-mo hyperoxia-treated rats were significantly reduced compared with week 3 and week 4 rats (all P < 0.04); minute phrenic activity responses in week 2 hyperoxia-treated rats appeared smaller than in week 4 rats, but this difference was not significant (P = 0.078).

Overall, hypoxic responsiveness in adult rats exposed to 1 wk of 60% O2 increased with increasing age of hyperoxic exposure, leveling off between weeks 3 and 4 (Fig. 2). Nonlinear regression of the change in minute phrenic activity at PaO2 = 40 Torr vs. age of exposure indicates a significant, positive relationship (second-order polynomial, P = 0.005, R2 = 0.39; Fig. 2). Although one rat in the week 4 group had a noticeably greater hypoxic response (383% increase from baseline) than other rats in this group, this has minimal influence on the regression analysis; removal of this data point would strengthen the relationship slightly (second-order polynomial, P = 0.002; R2 = 0.44).
DISCUSSION

Our laboratory previously reported that hypoxic phrenic responses are diminished in adult rats reared in hyperoxia (60% O2) for the first month of life (24). The present study extends this work, demonstrating that 1-wk hyperoxic exposures are sufficient to impair hypoxic phrenic responsiveness. However, the degree of functional impairment after 1 wk of hyperoxia decreases with advancing postnatal age. Whereas 1-wk exposures to 60% O2 during the third or fourth weeks of life had little impact on adult hypoxic responses, exposures during the first or second weeks were as effective as 1-mo hyperoxic exposures. Reduced impairment with advancing age is consistent with earlier reports that chronic hyperoxia in mature rats has no persistent effect on hypoxic ventilatory or phrenic responses (21, 24) or tyrosine hydroxylase staining in the petrosal ganglion (exposed at 21–28 days of age; Ref. 9). Collectively, these data indicate that there is a critical period during development during which hypoxic chemoreflexes are susceptible to hyperoxia. This “developmental window” appears to be open into the second postnatal week in rats.

To generate week 1 and 1-mo hyperoxia-treated rats, pregnant rats were placed into hyperoxia late in gestation (2–5 days before parturition), as in previous studies (12, 21, 23–25). Consequently, we cannot rule out an influence of prenatal hyperoxia on hypoxic phrenic responses. However, in rats exposed to hyperoxia during the second postnatal week only, blunted hypoxic responses were still observed. Thus prenatal exposure to hyperoxia is not necessary to impair adult hypoxic phrenic responses.

Hypoxic responsiveness was assessed by recording respiratory neural activity from the phrenic nerve, thereby allowing an assessment of neural mechanisms independent of changes in gas exchange or respiratory mechanics (24). Although phrenic activity is a neural analog of breathing, the present study did not measure ventilation per se, ignoring, for example, potential contributions of respiratory muscles other than the diaphragm. However, because developmental hyperoxia blunts both phrenic and ventilatory hypoxic responses (21, 24), reduced phrenic responses in this study are also likely to reflect blunted hypoxic ventilatory responses. In addition, bilateral vagotomy, used to prevent entrainment of phrenic bursts with the ventilator, eliminates some extracarotid chemoreceptor feedback (e.g., abdominal and aortic; Refs. 4, 28, 29). Nevertheless, vagotomy has no appreciable effect on the hypoxic phrenic responses of rats exposed to hyperoxia for the first month of life (24), suggesting that extracarotid chemoreceptors may also have been impaired by developmental hyperoxia.

Baseline PaCO2 levels were 2–3 Torr lower in rats exposed to hyperoxia during the first week or month of life relative to control, reflecting somewhat lower apneic thresholds in these groups. Similarly, in a previous study from our laboratory, rats exposed to 60% O2 for the first month of life also had lower baseline PaCO2 than age-matched controls (23). However, these same treatment groups did not differ significantly in other studies (12, 24, 25), so the importance of this observation is not clear. We attempted to standardize baseline phrenic motor output among preparations by controlling PetCO2 3 Torr above the apneic threshold. Higher PaCO2 levels may elevate baseline phrenic activity into a nonlinear range (8), thereby reducing the dynamic range available during hypoxic responses (10, 11, 18). On the basis of this reasoning, lower baseline PaCO2 would favor greater hypoxic phrenic responses in hyperoxia-treated rats rather than the attenuated responses actually observed (Refs. 12, 24, 25; present study). On the other hand, raising baseline PaCO2 toward control values could enhance hypoxic responses in hyperoxia-treated rats because of a positive interaction between hyperoxia and CO2 (15). Nevertheless, small differences in PaCO2 (2–3 Torr) are unlikely to account for the large (>50%) differences in hypoxic phrenic responses observed in the present study. Indeed, there was no apparent relationship between baseline PaCO2 and hypoxic responsiveness within or among treatment groups in this study (all P > 0.05; data not shown).

Critical periods in developmental plasticity. Many neural pathways are influenced by environmental conditions during early postnatal life (19). Thus some forms of plasticity are confined to critical periods of development when specific neural pathways are maturing. In developmental plasticity of the mammalian visual cortex, for example, monocular deprivation causes a persistent loss of visual function in the deprived eye but only if performed at critical developmental stages (16, 17). Although the specific age associated with this critical period varies across species, it corresponds to the same stage of visual cortex development (i.e., geniculocortical axons maturing to form ocular dominance columns) (17).

Considerable evidence implicates the carotid body, the principal source of hypoxic responsiveness in healthy mammals, as the site of impaired hypoxic responsiveness after chronic hyperoxia during development (7, 9, 12, 13, 22, 23). Similarly, exposure to 60% O2 for the first week of life has recently been found to attenuate carotid sinus nerve responses to cyanide, asphyxia, and hypoxemia in 3- to 5-mo-old anesthetized rats (50–75% of control responses; G. E. Bisgard, unpublished observations). The degree of carotid body functional impairment appears less severe after 1 wk of neonatal hyperoxia vs. 1 mo of hyperoxia (22), suggesting that 1-wk and 1-mo hyperoxic exposures could affect the hypoxic ventilatory response by different mechanisms. Although we cannot rule out additional peripheral or central effects of developmental hyperoxia, reduced carotid body chemosensitivity likely contributes to the blunted hypoxic phrenic responses observed in rats after 1-wk hyperoxic exposures.

The developmental window for impairment of the hypoxic phrenic response by 1-wk hyperoxic exposures (through the second postnatal week) corresponds to the period of carotid body maturation in rats (6). Rat ca-
rotid body responses to acute hypoxia are weak at birth and increase progressively during the first 2 wk of life (1, 20, 43). The correspondence between susceptibility to hyperoxia and carotid body maturation suggests that chronic hyperoxia may interfere with the normal, postnatal maturation of cellular O2 sensing in the carotid body. Alternatively, chronic hyperoxia may affect carotid chemoreflexes by altering synaptic connectivity to chemoafferent neurons. One week or one month of 60% O2 from birth causes long-lasting reductions in carotid body volume, partially through the loss of chemosensory tissue (9, 11a, 12), as well as severe loss of unmyelinated axons in the carotid sinus nerve (9). Chemoafferent neurons in the rat carotid sinus nerve require trophic support from their target tissue (i.e., the carotid body) during early postnatal life (14), although this dependence is no longer evident at 3 wk of age. Accordingly, Erickson and colleagues (9) proposed that hyperoxia may reduce the availability of trophic factors by reducing the amount of target tissue or by inhibiting the release of neurotrophins. These morphological changes in the carotid chemosensory pathway are associated with impaired chemoafferent responses to physiological stimulation (22), and their time course (9) is consistent with the developmental window reported here.

Other models of plasticity in hypoxic responses. Perinatal hypoxia in rats and sheep also causes changes in hypoxic ventilatory responsiveness that persist for weeks to months (33, 34, 38). For example, Okubo and Mortola (31, 33) reported greater resting ventilation and reduced hypoxic responsiveness in rats after chronic hypoxia during the first week of life (from 24 h through day 6), lasting until at least 50 days of age; similar effects were not observed in rats exposed to 6 days of hypoxia during the sixth week of life. The blunted hypoxic ventilatory response after perinatal hypoxia could involve changes in respiratory mechanics (32), but there is also considerable evidence that hypoxia delays maturation of carotid chemoreceptor function (13, 41). Although the developmental window for this functional impairment has not been determined, 1 wk of hypoxia early in the postnatal period is sufficient to alter future hypoxic responsiveness in rats (33), similar to the effects of hyperoxia.

Many mammalian species exhibit substantial plasticity after carotid body denervation, spontaneously recovering hypoxic ventilatory responsiveness (3, 26, 29, 36, 39). This recovery may involve upregulating or retaining functional aortic chemoreceptors (3, 26, 39) or, as observed in mature rats, upregulating abdominal and/or central hypoxia-sensitive tissues and chemosensory pathways (29, 36; also see Ref. 27). Whereas hypoxic ventilatory responses recover fully 45 days after carotid body denervation in male rats (36), hypoxic ventilatory and phrenic responses in vagally intact rats remain blunted at least 2–4 mo after hyperoxia during the first month of life (21, 24). These data suggest minimal upregulation of hypoxic chemoreceptor function after developmental hyperoxia, either because sufficient carotid chemoreceptor function remains to inhibit such upregulation or because the effects of hyperoxia are general to carotid body and other O2-sensitive chemoreceptors. The developmental window for potential impairment of extracarotid O2 chemoreceptors after perinatal hyperoxia remains to be investigated.

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